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(71) Applicants: BRIGHAM AND WOMEN'S HOSPITAL [US/US]; 75 Frances Street, Boston, MA 02115 (US). NEW ENGLAND DEACONESS HOSPITAL [US/US]; 185 Pilgrim Road, MASCO 302, Boston, MA 02215 (US).

(72) Inventors: NANJI, Amin; 38 Crestwood Drive, Wellsley, MA 02181 (US). STAMLER, Jonathan; 220 Marlborough Street, #1, Boston, MA 02118 (US). LOSCALZO, Joseph; 50 Pacella Drive, Dedham, MA 02026 (US).

(74) Agents: HERRON, Charles, J. et al.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US).

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(54) Title: THE USE OF NITRIC OXIDE-DELIVERING COMPOUNDS FOR THE TREATMENT OR PREVENTION OF ALCOHOLIC LIVER INJURY

(57) Abstract

Nitric oxide-delivering compounds are administered to an individual for the treatment or prevention of liver disease induced by ingestion of alcohol, or exposure to pharmacological agents or industrial toxins. Examples of nitric oxide-delivering compounds include S-nitrosothiols, thionitrites, thionitrates, sydnonimines, furoxans, organic nitrates, nitroprusside, nitroglycerine, iron-nitrosyl compounds, or other related compounds. In addition alcohol-induced liver disease may also be prevented by administering a therapeutically effective amount of either arginine, an arginine analog, or a nitric oxide-delivering compound, in combination with an alocholic beverage which is to be consumed by an individual.

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The Use of Nitric Oxide-Delivering Compounds For The Treatment Or Prevention Of Alcoholic Liver Injury

Background of the Invention

Field of the Invention

The invention relates to the use of nitric oxide-delivering compounds for the treatment or prevention of liver disease induced by alcohol or other hepatotoxins.

Brief Description of the Background Art

Chronic alcohol ingestion leads to a variety of serious disease states, the most wide spread of which is alcohol-induced liver disease. (Sultatos *et al.*, *Biochem. Physiol. of Substance Abuse, Vol. III*, CRC Press, Boca Raton, Florida (1991), pages 71-92). Cirrhosis, the end-stage of alcohol-induced liver disease, constitutes the eighth highest cause of death, and the fourth highest cause of death in males between 35 and 54 in the U.S. (Galambos, J., *Alcoholic Liver Disease*, Hall, ed., John Wiley & Sons, New York, (1985), page 230).

The liver serves as the most important biochemical regulatory center of the body. It is critical in the delivery and circulatory removal of substances such as glucose, galactose, fructose, ammonia, urea, vitamins, metals, free fatty acids, lipoproteins, coagulation factors, fibrinogen, globulins and albumin. In addition, the liver acts as a filter to clear potentially toxic endogenous and exogenous compounds from circulation, and facilitates the extraction and metabolism of substances originating in the digestive tract and

entering the portal circulation. Adequate liver function requires the continuous movement of molecules between hepatocytes and the blood stream; thus, a properly functioning vascular system is essential for optimal exchange efficiency.

Chronic exposure to alcohol leads to numerous metabolic and structural alterations within the liver which result in injury and consequential impairment of liver function. In particular, alcohol ingestion produces significant hepatic arterial vasoconstriction, impaired liver perfusion, and liver hypoxia. Vasoconstriction and hypoxia have been implicated in the pathogenesis of ethanol-induced hepatotoxicity, leading some investigators to suggest that alcoholic liver injury is very similar to ischemic heart disease (Thurman *et al., Fund. Appl. Toxocol., 4*:1250 (1984)).

It has been suggested that defective arginine synthesis may occur as a general response to alcohol and other hepatotoxins, and that it contributes to liver injury through the impairment of significant biochemical pathways. Trennery *et al., Toxicology Letters 19*:299-307 (1983). Arginine has an important role in all vascular systems because it serves as the substrate for the synthesis of nitric oxide by vascular endothelial cells (Palmer *et al., Nature 33*:664-666 (1988a)).

Synthesis of nitric oxide by the arginine pathway accounts for the endogenous vasodilatory action of endothelium-derived relaxing factor (EDRF). In addition to its significant role as an endogenous vasodilator, nitric oxide exerts cytostatic and cytotoxic actions against microbes and tumor cells, and prevents free radical-mediated injury, thrombocyte adherence and platelet aggregation. In experimental studies, hepatocytes subjected to chronic ethanol exposure exhibited a decreased capacity to produce nitric oxide, suggesting that nitric oxide deficiency is implicated in liver damage associated with alcohol ingestion (Silvio *et al.. Gastroenterology 102*(4 part 2):A801 (May 1992)).

In addition to alcohol, other hepatotoxins induce liver injury which is also characterized by arginine deficiency, vascular distortion, liver hypoxia, and the potential to progress to cirrhosis. For example, thioacetamide, a well known hepatotoxin, has been shown to significantly decrease plasma arginine levels in experimental animals (Trennery, *Toxicology Letters 19*:299-307 (1983)). Other chemical compounds such as carbon tetrachloride, are associated with an increase in orotic acid excretion, indicating a deficiency in arginine levels (Visek *et al.*, *J. Amer. Coll. Nutrition 5*:123-166 (1986)). Chronic exposure to a variety of other chemical compounds, such as lead, halogenated hydrocarbons, hexachlorobenzene, polychlorinated biphenyls, toluene, methylene chloride, turpentine, petroleum distillate, and other organic solvents, has also been implicated in liver injury.

Liver injury and cirrhosis induced by alcohol and other hepatotoxins are serious illnesses which require long-term medical supervision and careful management. At the present time, therapy of underlying liver disease is largely supportive, with specific treatment being directed to the particular complications. The lack of adequate therapy for advanced stages of alcoholic liver injury emphasizes the need for pharmaceutical agents which are more efficacious in treating advanced liver injury, and are also suitable for prophylaxis and early treatment during the fully reversible initial stages of diseases. Furthermore, given the significant role of nitric oxide deficiency in the pathogenesis of alcoholic liver injury, there is a need for pharmacological agents which can act directly to prevent or reverse liver injury resulting from nitric oxide deficiency.

Summary

Liver injury induced by alcohol and other hepatotoxins has been associated with the deficiency of nitric oxide, and it has been proposed that this deficiency plays a causal role in the pathogenesis of liver injury. Consequently, the inventors have conceived of a method for preventing or treating liver injury, comprising the administration of a nitric oxide-delivering compound to a patient in need of such treatment.

Therefore, the invention relates to a method for the treatment of liver disease, comprising administration of a therapeutically effective amount of nitric oxide-delivering compound to an individual afflicted with said liver disease. The invention also relates to a method for the prevention of liver disease, comprising the administration of a therapeutically effective amount of a nitric oxide-delivering compound to an individual at risk for acquiring said liver disease. Liver disease may be induced by alcohol ingestion or exposure of said individual to compounds other than alcohol which are hepatotoxic, such as a pharmacologic agent or industrial toxin.

The nitric oxide-delivering compound which is administered in the methods of the invention, comprises an S-nitrosothiol, S-nitroso-protein, thionitrate, thionitrite, sydnonimine, furoxan, organic nitrate, nitroprusside, nitroglycerine, iron-nitrosyl compound, or other related compound. Particular S-nitrosothiol compounds include S-nitroso-N-acetylcysteine, S-nitroso-cysteine, S-nitroso-homocysteine, S-nitroso-captopril, S-nitroso-glutathione, S-nitroso-pantathoine derivatives, S-nitroso-penicillamine, long-chain lipophilic nitrosothiols and S-nitroso-dithiols. Particular S-nitroso-proteins include S-nitroso-albumin.

The above methods also include administration of the nitric oxide-delivering compound as part of a pharmaceutical composition comprising one or more pharmaceutically acceptable carriers. Said pharmaceutical composition is administered to an individual by a route comprising

intravenous, intramuscular, subcutaneous, oral, topical, rectal, intranasal or aerosol delivery.

The invention also relates to a method for preventing alcohol-induced liver disease in an individual comprising administering to the individual, a therapeutically effective amount of a nitric-oxide delivering compound in combination with an alcohol-containing beverage. The invention further relates to a composition comprising an alcohol-containing beverage in combination with a therapeutically effective amount of a nitric oxide-delivering compound.

The invention also relates to a method for preventing alcohol-induced liver disease in an individual, comprising administering to an individual, a therapeutically effective amount of arginine or an arginine analog in combination with an alcohol-containing beverage. The invention further relates to a composition comprising an alcohol-containing beverage in combination with a therapeutically effective amount of arginine or an arginine analog. The arginine analog is selected from the group comprising arginine hydrochloride, N-α-benzoyl-L-arginine, N-α-benzoyl-L-arginine ethyl ester, L-citrulline, L-arginine methyl ester, D-phenylalanyl-L-propyl-L-arginine, N-α-benzoyl-L-arginine-ethylesterhydrochloride, poly-L-argininehydrochloride, NG-L-hydroxyarginine, arginine aspartate, N-omega-hydroxy-L-arginine.

Brief Description of the Drawings

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, appended claims and accompanying drawings where:

Figure 1 shows the liver pathology score for groups of animals fed a diet of either saturated fat plus ethanol, or corn oil plus ethanol, for one, two, or four week time periods;

Figure 2 shows the level of nitric oxide in non-parenchymal hepatic cells harvested from the livers of rats fed either saturated fat plus ethanol, or corn oil plus ethanol, over one, two, or four week time periods.

Description of the Invention

Definitions

To aid in the understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Treatment. By the term *"treatment"* is intended that the symptoms of the disease or pathological conditions which caused the disease be ameliorated or completely eliminated.

Prevention. By the term *"prevention"* is intended that the factor or factors which cause the disease be prevented.

Liver disease. By the term *"liver disease"* is intended any liver disorder which associated with decreased levels of plasma arginine or nitric oxide, and any impairment of liver function resulting therefrom, including impairment in the functioning of other bodily organs which occurs as the result of impaired liver function.

Individual. By the term *"individual"* is intended any living creature including mammals, and for example, humans.

Therapeutically effective. By the term *"therapeutically effective"* is intended an amount of the particular compound which when administered to an individual, is sufficient to treat or prevent the symptoms and/or factors which cause the liver disease.

Nitric oxide-delivering. By the term *"nitric oxide-delivering"* is intended any chemical compound capable of delivering *in vivo*, nitric oxide or an equivalent nitric oxide species in a biologically active form.

Alcohol. By the term *"alcohol"* is intended ethanol prepared in a form for human consumption.

Pharmacologic agent. By the term *"pharmacologic agent"* is intended a chemical compound intended for use in medical therapy.

Description of the Preferred Embodiments

The invention is based on the discovery by the inventors, that the treatment or prevention of liver disease can be accomplished by the administration of compounds which deliver nitric oxide *in vivo*, in a biologically active form. As demonstrated by the inventors, liver disease resulting from chronic ingestion of alcohol is associated with a decrease in nitric oxide levels in hepatic cells, and the decrease in nitric oxide has a significant causative role in the pathogenesis of liver injury. Thus, the invention provides a method for the treatment or prevention of liver disease by administering a nitric oxide-delivering compound to an individual afflicted with, or at risk of acquiring, liver disease.

One embodiment of the invention relates to a method for treating liver disease by administering a therapeutically effective amount of a nitric oxide-delivering compound to an individual afflicted with liver disease. While the inventors do not want to be limited to a particular theory regarding the mechanism by which nitric oxide-delivering compounds treat liver disease, it is proposed that by delivering nitric-oxide in a bioactive form, these compounds eliminate the nitric oxide deficiency caused by depleted levels of arginine. Once delivered, the nitric oxide causes vasodilation of constricted hepatic vessels, thereby improving liver perfusion and reducing liver hypoxia. In addition, these compounds act to reverse the abnormal platelet aggregation and other effects of endothelial damage caused by nitric oxide deficiency.

In another embodiment of the invention, nitric oxide-delivering compounds are administered to prevent the development of liver disease in an individual at risk for acquiring liver disease as a result of alcohol ingestion.

Administration of a nitric oxide-delivering compound to an individual at risk prevents nitric oxide deficiency, thereby preventing vasoconstriction, liver hypoxia, and other adverse consequences.

The above methods are useful for the treatment or prevention of liver disease which is induced by any alcohol ingestion which is sufficiently prolonged or excessive so as to cause the ingesting individual to experience a deficiency in plasma levels of arginine or nitric oxide. It should be recognized that the amount of alcohol required to induce these deficiencies will vary for each individual, depending on genetic predisposition, immune response, hormonal factors, environmental factors, nutrition, and other conditions which are associated with the alcohol ingestion. Individuals afflicted with alcohol-induced liver disease may exhibit a variety of clinical manifestations, ranging from asymptomatic or mild illness, to fatal hepatic insufficiency. To identify patients with alcohol-induced liver disease who are asymptomatic, or to confirm arginine or nitric oxide deficiency in patients exhibiting clinical symptoms, plasma arginine and nitric oxide levels may be monitored using standard assay techniques which are routine to those in the art (Stamler *et al.*, *Proc. Natl. Acad. Sci. USA, 89*:444-448 (1992); Stamler *et al.*, *Proc. Natl. Acad. Sci. USA, 89*:7674-7677 (1992); Stamler *et al.*, *Anal. Chem. 64*:779-785 (1992)).

Another embodiment of the invention relates to the administration of a therapeutically effective amount of a nitric oxide-delivering compound to an individual afflicted with liver disease or at risk of acquiring liver disease induced by exposure to a compound other than alcohol. These compounds include pharmacologic agents and industrial toxins which induce liver disease as a result of ingestion, inhalation, or topical or parenteral exposure.

Pharmacologic agents which may induce liver injury include, but are not limited to, acetaminophen, methyldopa, methotrexate, isoniazid, halothane, chlorpromazine, sodium valproate, oral contraceptives, tetracycline, and erythromycin. In addition, liver disease may be induced by the synergistic

effect of exposure to a pharmacologic agent in conjunction with alcohol ingestion.

Industrial toxins are those chemical agents which are used primarily in industrial processes, but may also be used by individuals during such activities as paint stripping, painting, and automobile repairs. Examples of such industrial toxins, include, but are not limited to lead, hexachlorobenzene, polychlorinated biphenyls, toluene, methylene chloride, turpentine, petroleum distillate, carbon tetrachloride, trichlorethylene, and yellow phosphorus, and other petro-chemicals.

Nitric oxide-delivering compounds which are useful in the methods of the invention include, but are not limited to S-nitrosothiols, S-nitroso-proteins, nitroprusside, thionitrites, thionitrates, iron-nitrosyl compounds, sydnonimines, furoxans, nitrosonium salts, and organic nitrates, and related compounds. Examples of S-nitrosothiols include, but are not limited to S-nitroso-N-acetylcysteine, S-nitroso-cysteine, S-nitroso-homocysteine, S-nitroso-captopril, S-nitroso-glutathione, S-nitroso-pantathoine derivatives, S-nitroso-penicillamine, long-chain lipophilic nitrosothiols, and S-nitroso-dithiols.

S-nitrosothiols are especially advantageous for use in the above methods of treatment or prevention of liver disease. These compounds have the capacity to deliver nitric oxide in a highly biologically relevant and non-toxic form, as nitrosonium or nitroxyl. The inventors conducted studies to assess the reactivity of S-nitrosothiols in the presence of redox metals. The studies demonstrated that S-nitrosothiols were not reactive with redox metal, thus indicating that the reactive nitric oxide species donated by the S-nitrosothiol is nitrosonimun or nitroxyl.

In addition, to causing vasodilation, the inventors have demonstrated that S-nitrosothiols increase the oxygen-binding capacity of hemoglobin, a globular protein, which binds reversibly to blood oxygen through passive diffusion from entry of air into the lungs. Hemoglobin-oxygen binding greatly increases the capacity of the blood to transport oxygen to bodily tissues.

Therefore, the binding affinity between hemoglobin and oxygen is a critical factor in determining the level of oxygen transferred to the tissues, and in particular, the tissues of the liver. Studies conducted by the inventors demonstrated that the reaction between S-nitrosothiols and hemoglobin produced a leftward shift in the hemoglobin-oxygen association curve, indicating a significant increase in oxygen binding. Therefore, the administration of S-nitrosothiols further facilitates the treatment or prevention of liver hypoxia by increasing the oxygen-carrying capacity in the blood, and consequential oxygen transport to the liver.

Particular S-nitroso-proteins include, but are not limited to S-nitroso-albumin. S-nitroso-proteins are particularly suitable for delivering nitric oxide *in vivo*. A unique advantage of these compounds is that they have the capacity to deliver nitric oxide at a slower rate, thus providing sustaining and long-lasting vasodilation in clinical situations which require such an effect. An additional advantage of these compounds is that they are much more easily assimilated physiologically because they are actually supplementing an already endogenous pool of bioactive nitric oxide. S-nitroso-albumin is an especially suitable compound for the delivery of nitric oxide. As with other S-nitroso-proteins, this compound produces a less rapid, but much more persistent vasorelaxant response, which is advantageous in counteracting vasoconstriction resulting from liver disease, and also to promote sustained liver perfusion and oxygen delivery to hepatic tissue.

Long carbon-chain lipophilic nitrosothiols are represented by the general formula $CH_3(CH_2)_xSNO$. S-nitroso-dithiols possess an additional thiol group, and are represented by the general formula $HS(CH_2)_xSNO$.

Examples of sydnonimines include, but are not limited to SYN-1 and 3- morpholinosydnonimine. Examples of organic nitrates include, but are not limited to, nitroglycerine, isosorbide dinitrate, erythritol tetranitrate and pentaerythritol tetranitrate. Iron-nitrosyl compounds are represented by the general formula $X_xFe_y(NO)_z$, wherein X is a low molecular weight or protein

thiol, or a non-thiolate anion such as phosphate, ascorbate, anionic protein, or glycosaminoglycan, such as heparin sulfate.

An additional embodiment of the invention relates to the methods of the invention in which the nitric oxide-delivering compound is administered as part of a pharmaceutical composition, comprising a pharmaceutically acceptable carrier.

The pharmaceutical compositions utilized in this invention can be administered by intranasal, aerosol, oral, enteral, topical, sublingual, rectal, intramuscular, intravenous, or subcutaneous means.

The compounds of this invention can be employed in combination with conventional excipients; i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral or intranasal application which do not deleteriously react with the active compounds. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohol, vegetable oils, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxymethylcellulose, polyvinylpyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavoring and/or aromatic substances and the like which do not deleteriously react with the active compounds. See generally, *Remingtons Pharmaceutical Science*, 16th edition, Mac Eds. 1980.

For parenteral application, particularly suitable vehicles consist of solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. Ampules are convenient unit dosages.

For enteral application, particularly suitable are tablets, dragees or capsules having talc and/or a carbohydrate carrier binder or the like, the

carrier preferably being lactose and/or corn starch and/or potato starch. A syrup, elixir or the like can be used wherein a sweetened vehicle is employed. Sustained release compositions can be formulated including those wherein the active component is protected with differentially degradable coatings, e.g., by microencapsulation, multiple coatings, etc.

It will be appreciated that the actually preferred amounts of active compounds used will vary according to the specific nitric oxide-delivering compound being utilized, the particular compositions formulated, the mode of application and the particular site of administration. Optimal administration rates for a given protocol of administration can be readily ascertained by those skilled in the art, using conventional dosage determination tests conducted with regard to the foregoing guidelines. A preferred dosage range is 1nmol/kg/minute to 1 mmol/kg/minute.

According to the present invention, a "therapeutically effective amount" of a pharmaceutical composition is an amount which is sufficient to achieve the desired pharmacological effect. Generally, the dosage required to provide an effective amount of the composition, and which can be adjusted by one of ordinary skill in the art, will vary, depending upon the age, health, physical condition, sex, weight and extent of disease, of the recipient. Additionally, the dosage may be determined by the frequency of treatment and the nature and scope of the desired effect. Appropriate dosages will be determined by those of ordinary skill in the art, using routine methods.

An additional embodiment of the invention relates to a method for preventing alcohol-induced liver disease by administering to an individual, a therapeutically effective amount of a nitric oxide-delivering compound in combination with an alcohol-containing beverage. The invention also includes compositions comprising an alcohol-containing beverage in combination with a therapeutically effective amount of a nitric oxide-delivering compound.

Compounds which are to be added to alcoholic beverages may be prepared in the form of a liquid, gel or solid, such as a powder, granules, or

a tablet. The compounds may be combined with acceptable carriers, including water, salt solutions, alcohol, oils, preservatives, stabilizers, emulsifiers, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously react with the arginine compound. Compounds may be suitable for addition to any ethanol-containing beverage which is prepared for human consumption. Suitable ethanol-containing beverages include non-distilled spirits, such as beers, wines and hard ciders, and distilled spirits, such as various types of brandy, cognac, rum, vodka, gin, whisky, bourbon, vermouth or mixed drinks containing one or more non-distilled or distilled spirits. The compounds may be suitable either for addition to the alcoholic beverage immediately prior to serving or prior to its packaging and storage.

Another embodiment of the invention relates to a method for preventing alcohol-induced liver disease in an individual, by administering to an individual, a therapeutically effective amount of arginine or an arginine analog in combination with an alcohol-containing beverage. A preferred dosage range is 15-100 grams per day. While the inventors do not want to be limited to a particular mechanism of action, it is proposed that by providing arginine or an arginine analog which is a substrate for nitric oxide synthesis, nitric oxide deficiency will be prevented.

Another embodiment of the invention includes pharmaceutical compositions comprising an alcohol-containing beverage in combination with a therapeutically effective amount of arginine or an arginine analog.

Examples of arginine analogs include, but are not limited to, arginine hydrochloride, N-α-benzoyl-L-arginine, N-α-benzoyl-L-arginine ethyl ester, L-citrulline, L-arginine methyl ester, D-phenylalanyl-L-propyl-L-arginine, N-α-benzoyl-L-arginine-ethylesterhydrochloride,poly-L-argininehydrochloride, NG-L-hydroxyarginine, arginine aspartate, N-omega-hydroxy-L-arginine.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following examples are, therefore, to be construed as merely

illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

The entire text of all publications cited above and below are hereby incorporated by reference.

Experimental

Example 1

Experiments were conducted,using animal groups fed either saturated fat (SF) in combination with ethanol (E), or corn oil (CO) in combination with ethanol, over 1, 2 or 4 week time periods. In animal models of experimentally-induced alcoholic liver injury, saturated fat prevents ethanol-induced liver disease, while corn oil, fed in conjunction with ethanol, induces liver injury (Nanji and French, *Life Sciences 44*:223-227 (1989)). Data pertaining to the degree of liver injury and nitric oxide levels in hepatocytes was obtained in order to demonstrate the relationship between liver injury and nitric oxide levels.

Animal Groups:

I. SF + E for 1 wk (n=5)
II. CO + E for 1 wk (n=5)
III. SF + E for 2 wks (n=4)
IV. CO + E for 2 wks (n=4)
V. SF + E for 1 month (n=5)
VI. CO + E for 1 month (n=5)

Histologic analysis

A small sample of liver was obtained at sacrifice and formalin-fixed. Hematoxylin and eosin stain was used for light microscopy. The examination

was carried out by a pathologist who had no prior knowledge of the dietary groups. The liver pathology was scored as follows: steatosis (the percentage of liver cells containing fat) -1+=0-25% of cells containing fat, 2+=26-50%, 3+=51=75%, 4+=>75; inflammation and necrosis - one focus/lobule = 1+, two or more foci/lobule =2+. The total liver pathology score was calculated by adding the scores from each of the parameters.

The results are shown in Figure 1. As shown, in animals fed CO+E, the pathology score was <1 after two weeks, 2+ after three weeks, and 4+ following four weeks of treatment.

Isolation of Non-parenchymal cells (NPC)

NPC were harvested from the livers of anaesthetized rats using isolation procedures and buffers previously described (Nanji *et al.*, *Gastroenterology 102*:A570 (1992)). In all experimental groups, NPC from 4-5 rats were combined. This allowed the collection of an adequate amount of NPC for stimulation by lipopolysaccharide. Briefly, after intravenous administration of sodium heparin, the livers were exsanguinated by hepatic artery perfusion with Ca^{2+} free buffer (0.01M HEPES with 0.39% NaCl - 0.05% KCl, Ph 7.4). Livers were excised, minced and incubated with 0.05% collagenase in buffer (0.1M HEPES with 0.83% NaCL - 0.05% Kcl - 0.05 M $CaCl_2$, Ph 7.6) at 37°C for 60 min. The resulting suspension was pelleted and reincubated with fresh collagenase buffer for 30 min. The cell suspension was pelleted and resuspended. Hepatocytes and cell clumps were removed by low speed centrifugation (500 g, 10 min. x 3). The remaining cells were washed with Gey's balanced salt solution. Final purification was achieved by centrifugation in a 17.5% solution of metrizamide in Gey's balanced salt solution.

With normal livers, the sinusoidal cell preparation contains 60-65% Kupffer cells following the gradient step; this is increased to 85-90% following adherence to plastic. In cases of diseased livers, the yield of Kupffer cells is

variable and depends on degree of fat infiltration, necrosis and inflammation. However, in no preparation was the content of hepatocytes greater than 1%. Two suspensions of $5X10^7$ cells/ml in RPMI 1640 with 5% fetal bovine serum were placed in 35-cm^2 tissue culture dishes and incubated overnight at 37°C. The next day, 200 ng/ml of lipopolysaccharide (E. coli J5 mutant of $0111B_4$ serotype) was added to the cell suspensions. After 3 hours, the supernatants of cell suspensions were removed by centrifugation and stored at - 70°C for analysis of nitrites.

Nitrite (NO) was detected using previously described methods (Stamler *et al.*, *Proc. Natl. Acad. Sci. USA, 89*:7674-7677 (1992), Stamler *et al.*, *Proc. Natl. Acad. Sci. USA, 89*:444-448 (1992), and Stamler *et al.*, *Anal. Chem. 64*:779-785 (1992), and Saville, *Analyst 83*:670-672 (1958)). As shown in Figure 2, nitrite levels were lowest in the hepatocytes of animals with the greatest degree of liver pathology. In general, the decrease in nitric oxide was directly proportional to the increase in liver pathology. For example, in animals fed CO + E for four weeks, the concentration of nitrite was 2.8 millimolar, while in animals fed SF + E for four weeks, it was 17.0 millimolar. These results demonstrate that ethanol-induced liver injury is clearly associated with nitric oxide deficiency.

What Is Claimed:

1. A method for the treatment of liver disease, comprising the administration of a therapeutically effective amount of a nitric oxide-delivering compound to an individual afflicted with said liver disease.

2. A method for the prevention of liver disease, comprising the administration of a therapeutically effective amount of a nitric oxide-delivering compound to an individual at risk for acquiring said liver disease.

3. The method of claim 1 or 2 wherein said liver disease or risk of said liver disease is induced by alcohol ingestion by said individual.

4. The method of claim 1 or 2 wherein said liver disease or risk of said liver disease is induced by exposure of said individual to a compound other than alcohol.

5. The method of claim 1 or 2 wherein said liver disease is induced by exposure of said individual to a pharmacologic agent.

6. The method of claim 5 wherein said pharmacologic agent comprises acetaminophen, methyldopa, methotrexate, isoniazid, halothane, chlorpromazine, sodium valproate, and oral contraceptives.

7. The method of claim 1 or 2 wherein said liver disease is induced by exposure of said individual to an industrial toxin.

8. The method of claim 6 wherein said industrial toxin comprises lead, hexachlorobenzene, polychlorinated biphenyls, toluene, methylene chloride, turpentine, petroleum distillate, carbon tetrachloride, trichlorethylene, and yellow phosphorus.

9. The method of claim 1 or 2 wherein said nitric oxide-delivering compound comprises an S-nitrosothiol, S-nitroso-protein, thionitrite, thionitrate, iron-nitrosyl compound, nitroglycerine, sydnonimine, furoxan, organic nitrate, nitroprusside, or other related compound.

10. The method of claim 9 wherein said S-nitrosothiol compound is selected from the group comprising S-nitroso-N-acetylcysteine, S-nitroso-cysteine, S-nitroso-homocysteine, S-nitroso-captopril, S-nitroso-glutathione, S-nitroso-pantathoine derivatives, S-nitroso-penicillamine, long-chain lipophilic nitrosothiols, and S-nitroso-dithiols.

11. The method of claim 1 or 2 wherein said compound is administered as part of a pharmaceutical composition comprising a pharmaceutically acceptable carrier.

12. The method of claim 11 wherein said pharmaceutical composition is administered to an individual by a route comprising intravenous, intramuscular, subcutaneous, oral, topical, rectal, intranasal, or aerosol delivery.

13. The method of claim 1 or 2 wherein said individual is a human.

14. A method for preventing alcohol-induced liver disease in an individual comprising administering to the individual, a therapeutically effective

amount of a nitric oxide-delivering compound in combination with an alcohol-containing beverage.

15. A method for preventing alcohol-induced liver disease in an individual, comprising administering to the individual, a therapeutically effective amount of arginine or an arginine analog in combination with an alcohol-containing beverage.

16. A composition comprising an alcohol-containing beverage in combination with a therapeutically effective amount of arginine or an arginine analog.

17. The method of claim 15 or 16 wherein said arginine analog comprises arginine hydrochloride, N-α-benzoyl-L-arginine, N-α-benzoyl-L-arginine ethyl ester, L-citrulline, L-arginine methyl ester, D-phenylalanyl-L-propyl-L-arginine, N-α-benzoyl-L-arginine-ethyl ester hydrochloride, poly-L-arginine hydrochloride, NG-L-hydroxyarginine, arginine aspartate, N-omega-hydroxy-L-arginine.

18. A composition comprising an alcohol-containing beverage in combination with a therapeutically effective amount of nitric oxide-delivering compound.

19. The method of claim 18 wherein said nitric oxide delivering compound comprises an S-nitrosothiol, S-nitroso-protein, thionitrite, thionitrate, iron-nitrosyl compound, nitroglycerine, sydnonimine, furoxan, organic nitrate, nitroprusside, or other related compound.

20. The method of claim 19 wherein said S-nitrosothiol compound is selected from the group comprising S-nitroso-N-acetylcysteine, S-nitroso-cysteine, S-nitroso-homocysteine, S-nitroso-captopril, S-nitroso-glutathione, S-nitroso-pantathoine derivatives, S-nitroso-penicillamine, long-chain lipophilic nitrosothiols, and S-nitroso-dithiols.

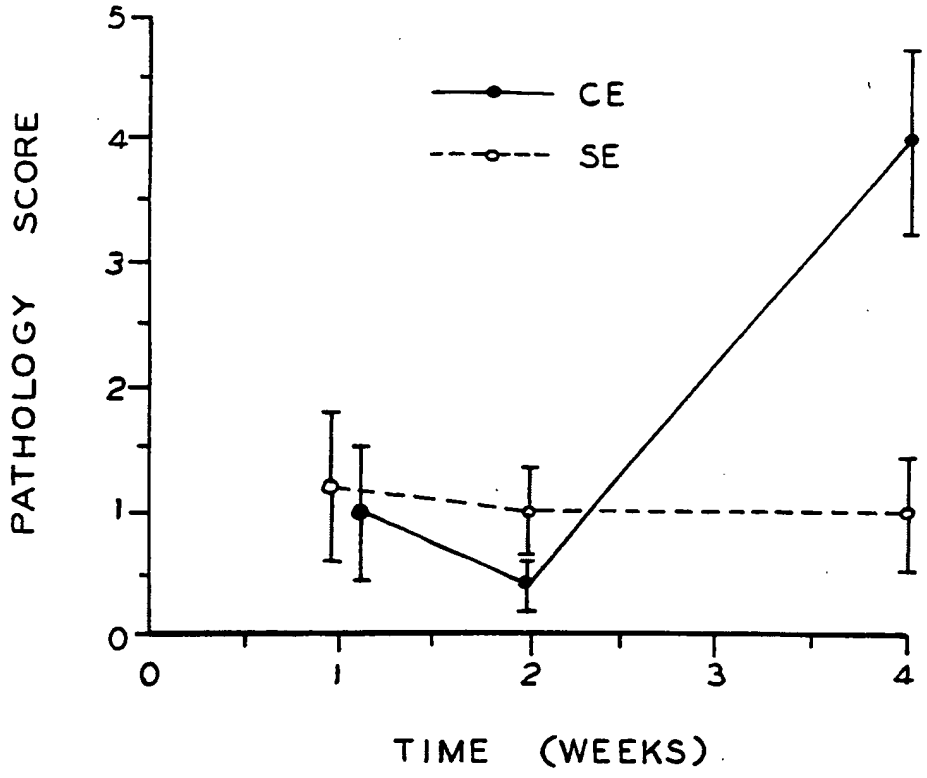
PATHOLOGY SCORE
5
4
3
2
1
0
CE
SE
0
1
2
3
4
TIME (WEEKS)

FIG. 1

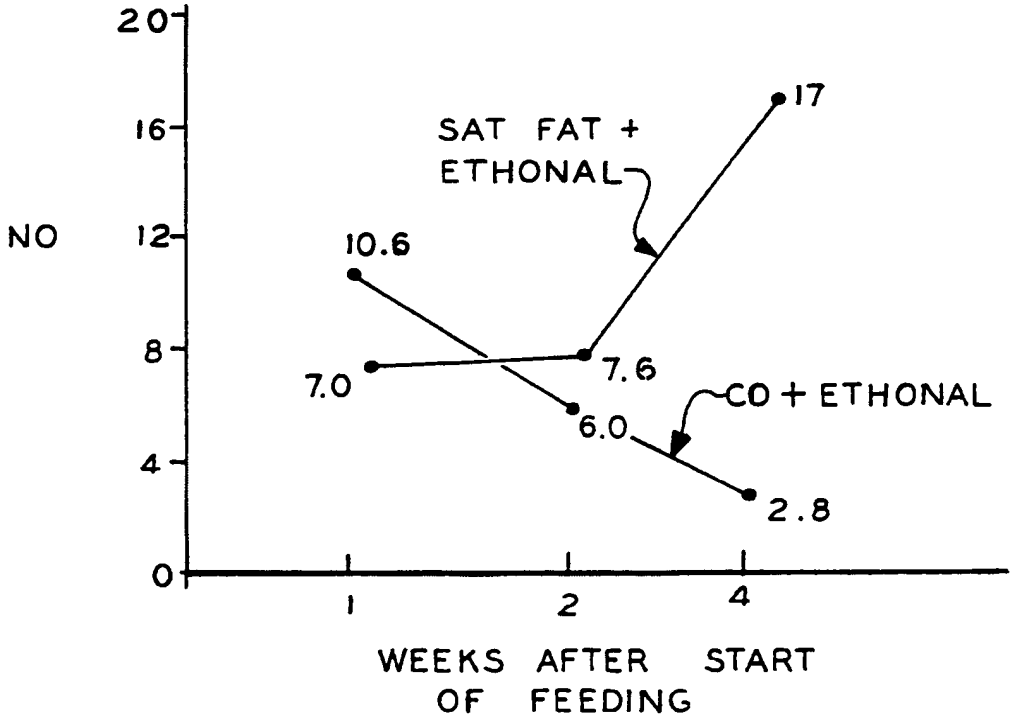
NO
20
16
12
8
4
0
SAT FAT + ETHONAL
17
10.6
7.0
7.6
CO + ETHONAL
6.0
2.8
1
2
4
WEEKS AFTER START OF FEEDING

FIG. 2

INTERNATIONAL SEARCH REPORT

Int tional application No.
PCT/US94/00970

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 49/00, 31/195
US CL :424/10; 514/563, 565

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/10; 514/563, 565

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Please See Extra Sheet.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US, A 4,987,123 (MASAKI ET AL) 22 JANUARY 1992, COLUMN 1, LINE 28 AND LINES 13-15	1-5,7,11-13 ---------- 1-20
Y	US, A, 3,950,529 (FISCHER ET AL) 13 APRIL 1976, SEE ENTIRE DOCUMENT, ESPECIALLY COLUMN 3, LINES 49, 63; COLUMN 1, LINE 43,44, AND ABSTRACT	1-20
Y	R. BERKOW ET AL., "THE MERCK MANUAL OF DIAGNOSIS AND THERAPY" 14TH EDITION, PUBLISHED 1982 BY MERCK SHARP & DOHME RESEARCH LABORATORIES (N.J.), SEE ESPECIALLY PAGES 830-833 AND 846-849	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 01 APRIL 1994	Date of mailing of the international search report MAY 16 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. 703-305-3230	Authorized officer GREGORY HOOK Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

Int ional application No.
PCT/US94/00970

B. FIELDS SEARCHED
Documentation other than minimum documentation that are included in the fields searched:

THE MERCK MANUAL OF DIAGNOSIS AND THERAPY, 14TH EDITION, ED., BERKOW ET AL., PUB . MERCK SHARP & DOHME RESEARCH LABORATORIES, 1982